ISOLATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR OF ALFALFA

by

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B. S., Chung-Hsin University, 1959

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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Manhattan, Kansas

1969

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INTRODUCTION

Trypsin-inhibiting substances are very widely distributed in nature. They have found in many animal and plant sources. They first were observed in Ascaris (1, 2), and egg white (3) at the beginning of this century. Although they were known such a long time ago, study of them progressed very slowly due to the fact that knowledge about enzymology and inhibitors was limited.

During the thirty years following their discovery, little progress was made in the study of tryptic inhibitors. Interest was aroused in 1931 when Northrop and Kunitz reported their efforts to crystallize trypsin and one of its inhibitors (4, 5, 6). Thereafter, this inhibiting substance, which had been a matter of speculation for so long, now was available in crystalline form. Subsequently, many tryptic inhibitors, from different sources, have been isolated and characterized. In some cases, the results of different authors did not agree because they performed experiments with impure inhibitors. More recently, by using ion-exchange column for more extensive purification, these discrepancies have been eliminated (7, 8, 9). The introduction of column techniques has been very important in tryptic inhibitor fractionation. By using them, many inhibitors have been isolated in pure form (7-11).

Trypsin inhibitors isolated from natural sources, in most cases, are glycoproteins. The α-helix content is quite low. The structure is largely tertiary in character (12, 13). The inhibitory action with trypsin originally was thought to involve formation of an inactive complex between trypsin and the inhibitor with secondary interaction (14). This mechnism has been shown to be incorrect. Actually, the complex formation involves a cleavage of an especially sensitive bond in the inhibitor by trypsin and then formation of a

covalent bond subsequently between trypsin and the inhibitor (15-22). The sensitive bond has been demonstrated to involve either lysine or arginine. This lead Haynes and Feeney (23) to classify tryptic inhibitors into two groups:

Lysine inhibitors

1. Lima bean trypsin inhibitor
2. Bovine colostrum
3. Turkey ovomucoid
4. Duck ovomucoid
5. Cassowary ovomucoid
6. Penguin ovomucoid

Arginine inhibitors

1. Chicken ovomucoid
2. Soybean trypsin inhibitor

The inhibitory activity of naturally occurring trypsin inhibitors follows second-order kinetics (23-25). However, it is much more complicated. It actually involves a series of Michaelis-type complexes with continuous conformation changes (23). The inhibitor never seems to exert its maximum theoretical effect against trypsin. There is an equilibrium existing between the virgin and the modified inhibitor (26).

The metabolic effects caused by trypsin inhibitor include depression of growth and reduction in food utilization (27). These may be related to reduced protein digestion resulting from the enzyme-inhibitor complex formation (28). Furthermore, the activities of other enzymes such as xanthine dehydrogenase, xanthine oxidase and arginase decrease significantly in the presence of trypsin inhibitor (29).

The utilization of methionine and cystine also is affected by trypsin inhibitor. Kwong et al (30) reported that in the presence of soybean trypsin inhibitor an increased oxidation of methionine is undoubtedly related to an increased conversion of methionine to cysteine. The increased cystine requirement is due to a metabolic block in its utilization for protein synthsis, and methionine supplementation acts both to provide more cystine and to make up the specific deficiency of methionine in protein.

Trypsin inhibitors of plant origin have been studied intensively in recent years. The presence of a trypsin inhibitor in alfalfa seeds was reported by Borchers et al (31). Kendall (32) also demonstrated trypsin inhibition by aqueous extracts of fresh alfalfa forage. Furthermore, Mitchell et al (33, 34) and mooijman showed a similar inhibition of trypsin by extracts of commercial dehydrated alfalfa.

The inhibitor isolated by Ramirez and Mitchell (34) appeared to be a polypeptide or a non-coagulable protein which is slowly heat labile, while the one obtained by mooijman (35) was a saponin-amino acid or a saponin-peptide complex which was extremely thermostable and stable over a wide pH range, at least between pH 2 and pH 12. Obviously, these two are different inhibitors. thus, it appears that more than one inhibitor can be found in a single source.

Alfalfa is an important source of protein and carotenoids for animal rations in the United States. The trypsin inhibitor might be partially responsible for growth depression which has been observed when alfalfa meal is added to chick rations at more than a ten percent level (27, 34). Thus, because of its possible nutritional effects, the trypsin inhibitor of alfalfa seems worthy of further investigation.

EXPERIMENTAL

Assay of Trypsin Activity

The proteolytic activity of trypsin was determined by a modification of the method of Beauchene et al (36), and Ramirez et al (34).

A three percent solution of casein in 0.107 M phosphate buffer, pH 8.4, was prepared and adjusted to pH 8.4 with dilute sodium hydroxide. A trypsin solution containing 30 mg. of trypsin in 100 ml. of 0.107 M phosphate buffer, pH 8.4, was prepared just before use. The assay mixture consisted of 5 ml. of the casein solution and 2 ml. of the trypsin solution. A final volume of 15 ml. was obtained by adding either distilled water or inhibitor solution. The final assay mixture was 0.05 M with respect to phosphate.

The reaction mixture was placed in a water bath maintained at 37° C.. After four hours, 5 ml. of 10 percent trichloroacetic acid were added to precipitate the trypsin and the undigested casein. The sample was filtrated and the filtrate was adjusted to pH 7 with 40 percent sodium hydroxide. Five ml. of the filtrate were placed in a 15 ml. graduated centrifuge tube and 5 ml. of a suspension of copper phosphate in pH 9.1 borate buffer (36) were added. The contents of the tube were mixed well, allowed to stand for 5-10 minutes and clarified by centrifugation. The absorbance of the supernatant solution was measured at 620 mu with a Beckman DU Spectrophotometer. A standard curve (Fig. 1) was prepared by carrying known amounts of alanine through the procedure.

Protein Determination

The protein contein of the isolated inhibitor was determined with Miller's

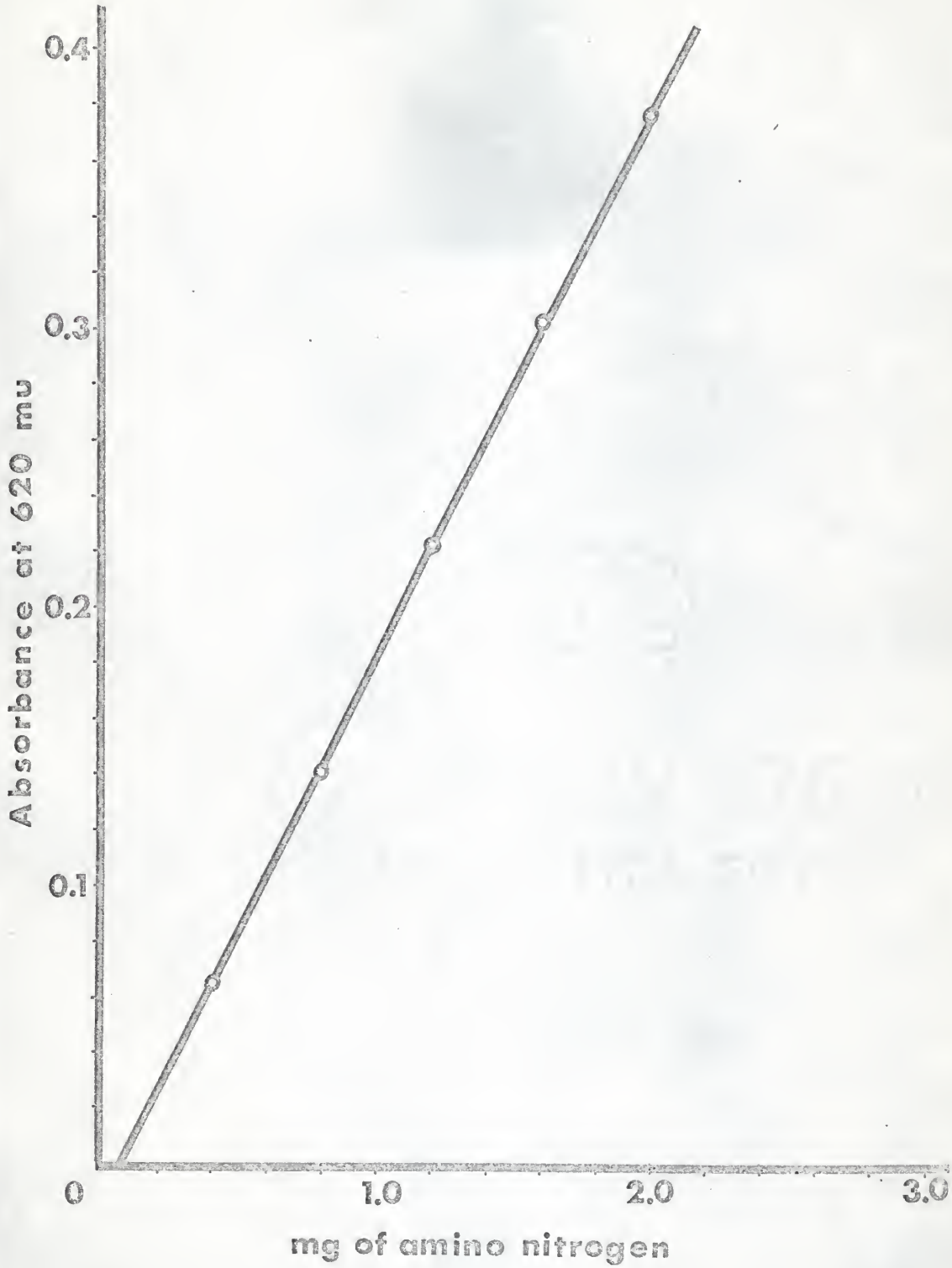

Fig. 1. Standard curve for assay of trypsin activity.

(37) modification of the Folin-Lowry method (38, 39).

Folin reagent was prepared by mixing 100 g. sodium tungstate, 25 g. sodium molubdate, 700 ml. water, 50 ml. 85 percent phosphoric acid and 100 ml. concentrated hydrochloric acid and refluxing gently for ten hours. Then 150 g. lithium sulfate, 50 ml. water and a few drops of liquid bromine were added. The solution was boiled 15 minutes to remove the excess bromine, cooled, diluted to 1000 ml., and filtered through a sintered glass funnel. The reagent was stored in a refrigerator and protected from any potential reducing materials. One part of this solution was mixed with ten parts of distilled water before use.

Alkaline copper reagent was prepared from two solutions:

Solution A-- 0.2 percent $CuSO_4.5H_2O$ in 0.6 percent tris(hydroxymethyl)-aminomethane.

Solution B-- 10 percent Na_2CO_3 in 0.5 N NaOH.

A small quantity of alkaline copper reagent was prepared as needed by adding one part of solution A to ten parts of solution B. This combined solution has a useful life of three days.

Miller's modification method was carried out by adding 1.0 ml. of alkaline copper reagent to 1.0 ml. of protein solution. The solution was mixed and allowed to stand for 10 minutes. Then, 3.0 ml. of Folin reagent were added by rapidly blowing most of the contents from a 3-ml. volumetric pipet directly into the tube and mixing. The last of the reagent was allowed to drain from the pipet as usual to complete the 3 ml. addition. After mixing, the tube was placed in a water bath at approximately 50° C. for 10 minutes. The solution was cooled to room temperature and its absorbance was measured at 650 mu. A blank was prepared by replacing the protein solution with water.

A solution containing 200 γ/ml. of bovine serum albumin was used to prepare the standard curve which is shown in Fig. 2.

Fractionation of Trypsin Inhibitor

Extraction.

Two Kg. of commercial dehydrated alfalfa meal were defatted by extraction with 8 l. of acetone and then three times with 8 l. of 80 percent aqueous ethanol. The defatted alfalfa was dried at room temperature for a few days. Ten l. of 0.25 N sulfuric acid were added and the mixture was allowed to stand overnight. The extract was collected by filtration through a piece of cheese cloth and was clarified by centrifugation at 2000 g. for 20 minutes. The inhibitor was precipitated from the clear extract by adding solid ammonium sulfate to 70 percent saturation. After standing overnight, the precipitate was recovered by centrifugation at 4000 g. for 10 minutes. The precipitate was dissolved in a minimum amount of distilled water. The solution was dialyzed against running tap water for 48 hours and then was equilibrated with distilled water. Finally, the extract, which will be referred to as " crude preparation", was frozen and kept in that state while awaiting further fractionation by column chromatography.

DEAE-cellulose column chromatography.

The DEAE-cellulose column was prepared according to the method of Peterson and Sober (40). The adsorbent was allowed to sink into a 1 N NaOH solution. The suspension was filtered and washed with 1 N NaOH solution until all color was removed. This was followed by the addition of 1 N HCl to make a strongly acidic suspension. Immediately after the addition of acid, the suspension was filtered. The cake then was resuspended in 1 N NaOH, the suspension was filtered, washed free of alkali with distilled water, and finally suspended in

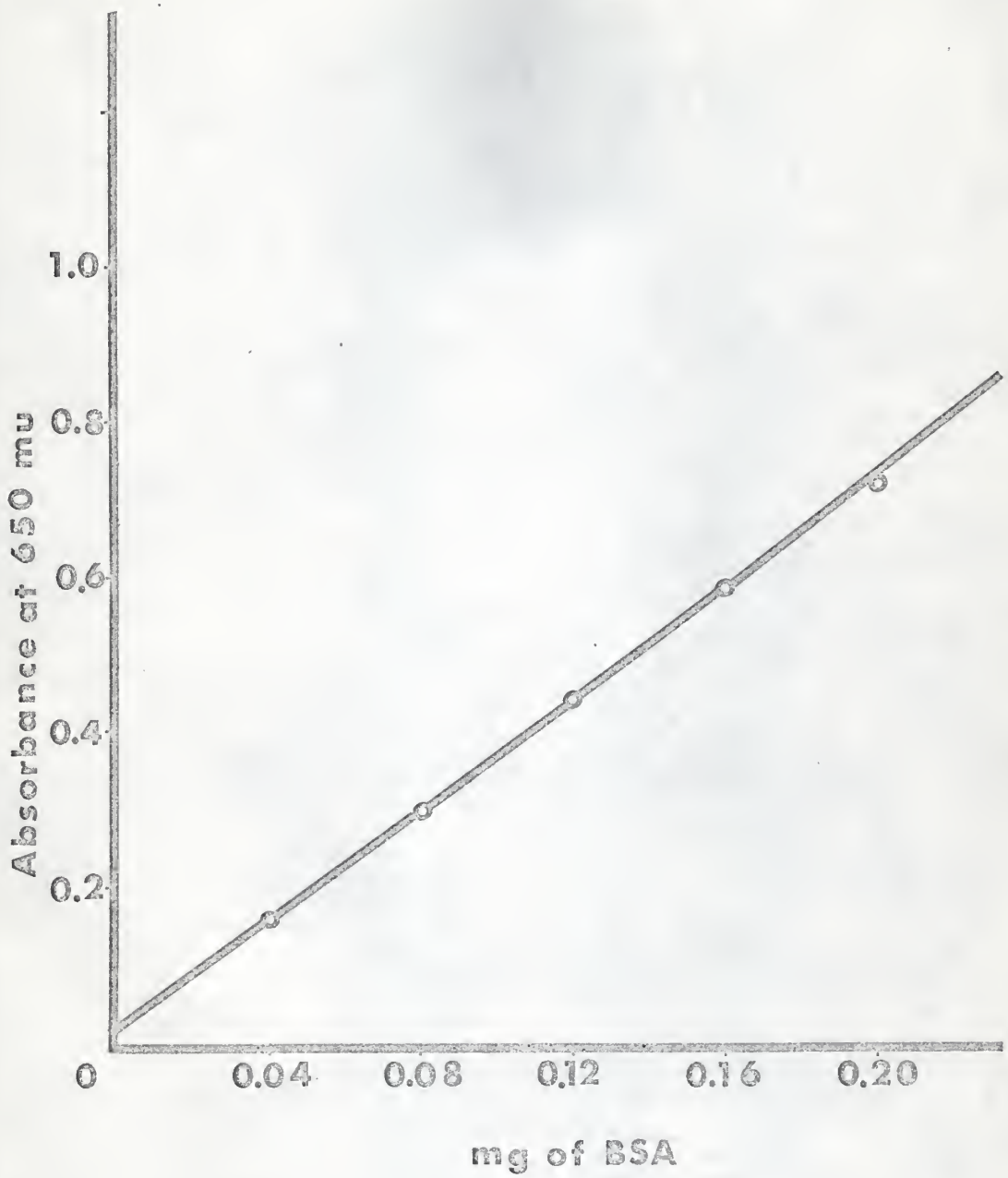

Fig. 2. Standard curve for spectrophotometric determination of protein.

the staring buffer.

A chromatographic column was prepared by covering a one-hole rubber stopper with a piece of 400-mesh nylon and forcing the stopper into one end of a 12 X 1 inch glass tube. A delivery tube was inserted into the hole of the rubber stopper. The adsorbent suspension was poured into the column until the final length of the adsorbent bed was 9 inches. The crude preparation, which had been equilibrated in the starting buffer and centrifuged, was placed on the column and the column was eluted with NaCl and 0.01 M citrate buffer pH 3.0, using an open volume NaCl gradient design (41, 42). The elute was collected in 5 ml. fractions, and the absorbance of each fraction was measured with a Beckman DU Spectrophotometer at 280 mu with a 0.6 mm slit.

The elute pattern is shown in Fig. 3. Fractions were combined in such a manner that peaks A, B, C, and D were contained in separate solutions. These were dialyzed and then concentrated by evaporation in the dialysis tubing hung in front of an electric fan. The inhibitory activity of each solution, and the crude preparation was measured. The protein content of each fraction was determined. The data for inhibitory activity and protein content are shown in Table I and Table II.

From these data, it is obvious that only the crude preparation and peak A were inhibitory. Therefore, additional amounts of peak A were collected by chromatographic separation of additional portions of the crude preparation and were used in further purification studies.

The fold of purification of peak A by DEAE-cellulose column can be calculated as follows:

$$\text{Fold} = \frac{\text{(amino nitrogen decrease/mg. of protein) of peak A}}{\text{(amino nitrogen decrease/mg. of protein) of crude preparation}}$$

where "amino nitrogen decrease" is the difference between amino acid released

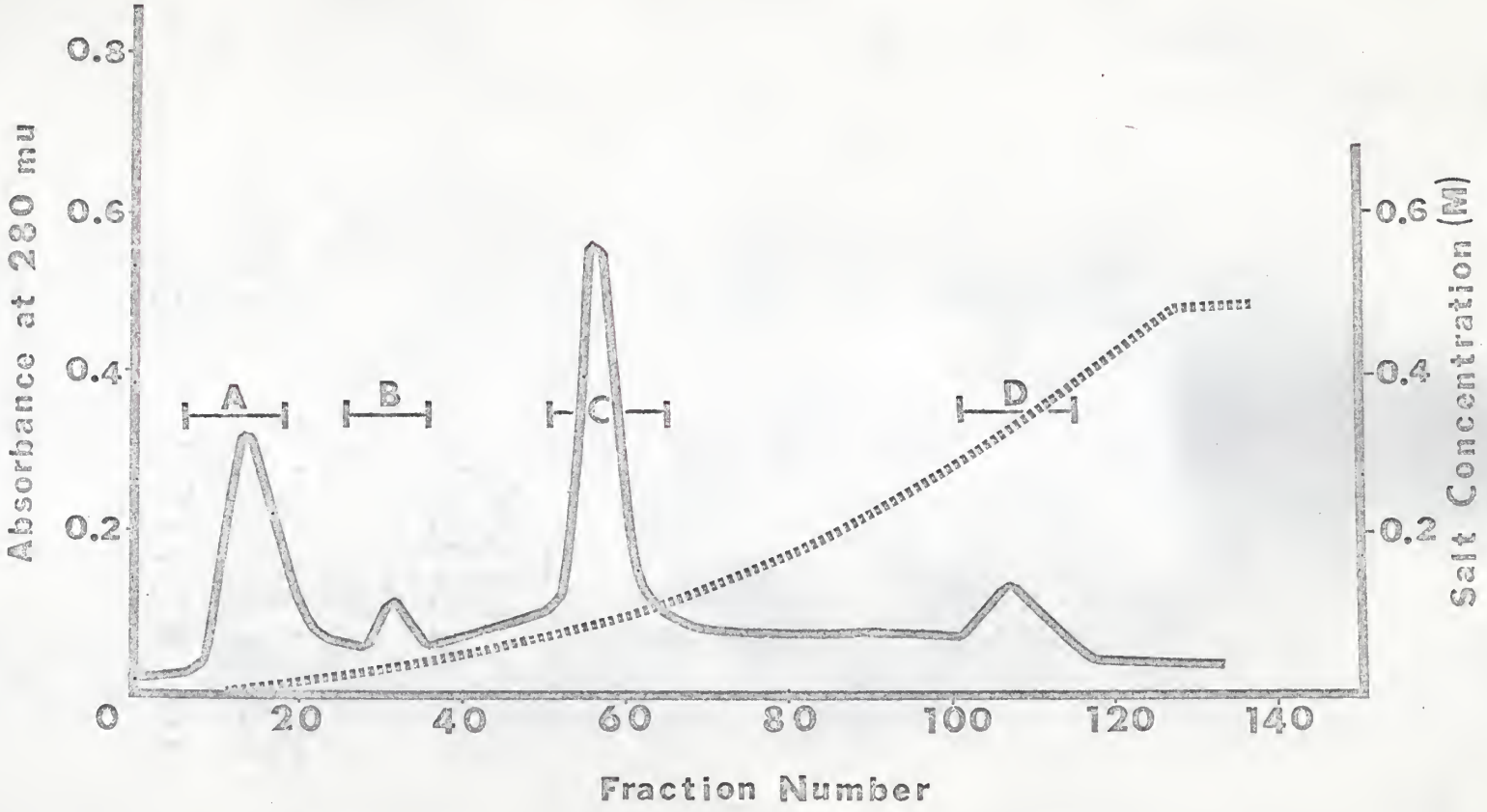

Fig. 3. Chromatographic pattern of DEAE-cellulose column. Elution was achieved by 0.01 M citrate buffer pH 3. The broken line indicates increasing salt concentration.

Table I. Inhibitory activity of the crude inhibitor preparation and of its fractions obtained by DEAE-cellulose column chromatography at pH 3.0.

Fraction	ml. used in assay	Trypsin solution	Absorbance at 620 mu	Net absorbance	Amino nitrogen released (mg)	Amino nitrogen decrease (mg)	Amino nitrogen decrease (mg per ml of fraction)
none		heated	0				
"		unheated	0.344	0.344	1.84		
crude preparation	5	heated	0.030				
"	5	unheated	0.270	0.240	1.30	0.54	0.108
peak A	8	heated	0				
"	8	unheated	0.252	0.252	1.36	0.48	0.060
peak B	8	heated	0				
"	8	unheated	0.342	0.342	1.82	0.02	0.003
peak C	8	heated	0				
"	8	unheated	0.342	0.342	1.82	0.02	0.003
peak D	8	heated	0				
"	8	unheated	0.343	0.343	1.84	0	0

Table II. Protein content of the crude inhibitor preparation and of its fractions obtained by DEAE-cellulose column chromatography at pH 3.0

Fraction	ml. used in assay	Absorbance at 650 mu	mg of protein per ml
none	0	0	0
crude preparation	0.2	0.329	0.440
peak A	1.0	0.258	0.068
peak B	1.0	0.051	0.008
peak C	1.0	0.131	0.032
peak D	1.0	0.044	0.066

from casein by trypsin and the amonut released by trypsin in the presence of the inhibitor.

The following data are from Table I and Table II:

mg. amino nitrogen decrease caused by 1 ml. of peak A added = 0.060

mg. of protein per ml. of peak A = 0.068

mg. amino nitrogen decrease caused by 1 ml. of crude preparation added = 0.108

mg. of protein per ml. of crude preparation = 0.440

Therefore, the fold of purification of peak A is:

$$\frac{0.060}{0.068} \div \frac{0.108}{0.440} = 3.6$$

Since peak A was the only fraction which was inhibitory, an effort was made to test its homogeneity by disc electrophoresis. The experiment was performed at pH 8.2-8.4 with a Canalco Model 6 Disc Electrophoresis apparatus by following the instruction manual prepared by the manufacturer, Canal Industrial Corporation, Bethesda, Md.. As shown in Plate I, there were four bands on the electrophoresis gel. Thus, further fractionation was necessary to achieve greater purity.

The peak A fraction was dialyzed, concentrated by evaporation in the dialysis tubing by hanging in front of an electric fan and equilibrated in the buffer which was to be used as the eluting agent described below. The fraction then was rechromatographed on a DEAE-cellulose column and eluted with NaCl and 0.05 M Tris-HCl buffer, pH 8.2. A stepwise NaCl gradient elution was used. The chromatographic pattern is shown in Fig. 4. Fractions A' to H' were collected and their inhibitory activities and protein contents were determined in the same manner as described earlier. The data are shown in Table III and Table IV. From these two tables, it is apparent that the

EXPLANATION OF PLATE I

Disc electrophoresis pattern of peak A obtained by DEAE-cellulose column chromatography.

PLATE I

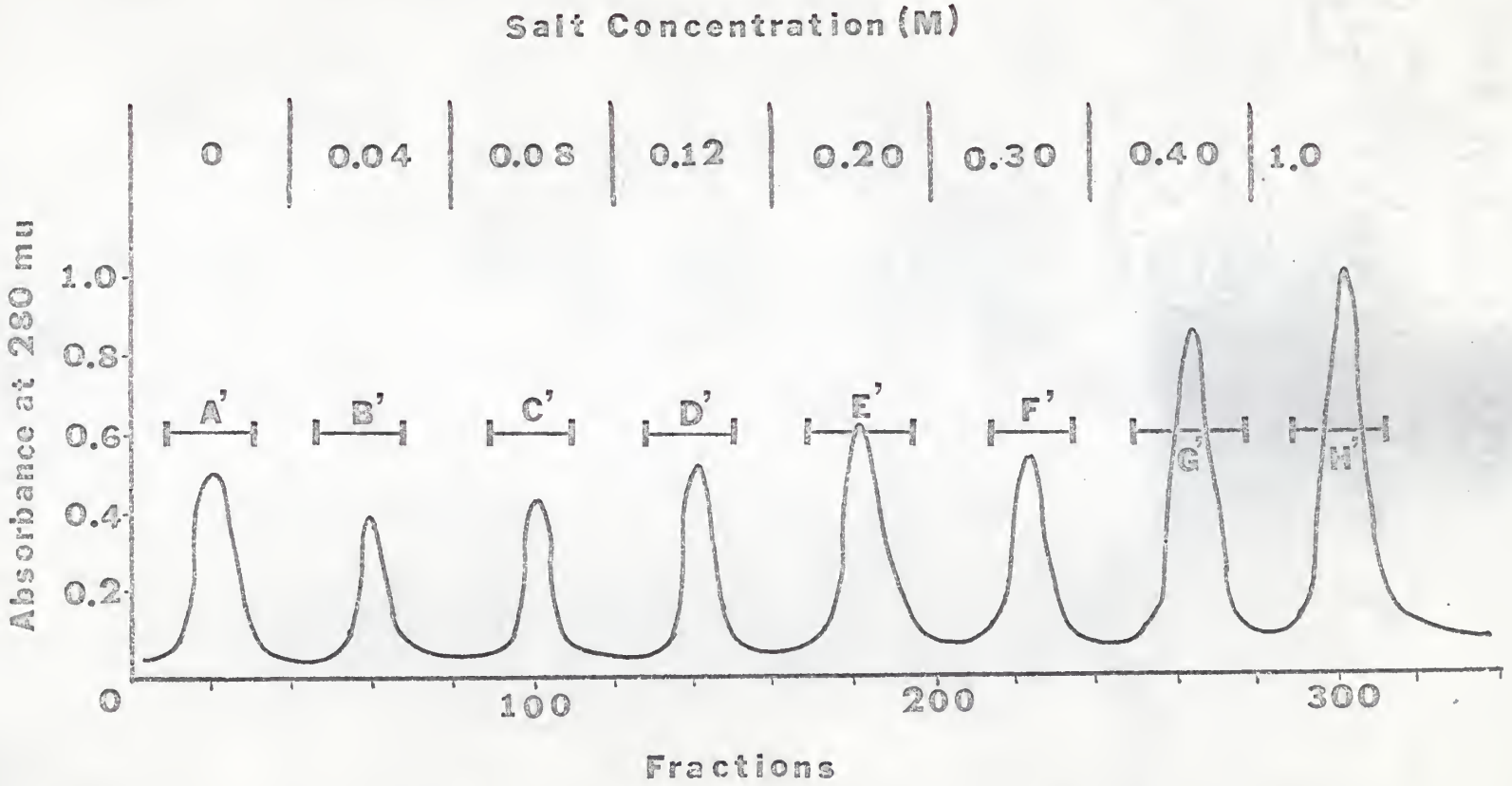

Fig. 4. Chromatographic pattern of DEAE-cellulose column. Elution was with 0.05 M Tris-HCl buffer pH 8.2 and salt gradient as shown.

Table III. Inhibitory activity of peak A and various fractions obtained from it by DEAE-cellulose column chromatography at pH 8.2.

Fraction	ml. used in assay	Trypsin solution	Absorbance at 620 mu	Net absorbance	Amino nitrogen released (mg)	Amino nitrogen decrease (mg)	Amino nitrogen decrease (mg per ml of fraction)
none		heated	0				
"		unheated	0.255	0.255	1.38		
peak A	4	heated	0.006				
"	4	unheated	0.178	0.172	0.95	0.43	0.108
peak A'	8	heated	0.002				
"	8	unheated	0.170	0.168	0.92	0.46	0.058
peak B'	8	heated	0.003				
"	8	unheated	0.225	0.222	1.21	0.17	0.021
peak C'	8	heated	0.004				
"	8	unheated	0.234	0.230	1.24	0.14	0.017
peak D'	8	heated	0.003				
"	8	unheated	0.232	0.229	1.24	0.14	0.017
peak E'	8	heated	0.003				
"	8	unheated	0.238	0.235	1.27	0.11	0.014
peak F'	8	heated	0.004				
"	8	unheated	0.240	0.236	1.28	0.10	0.012
peak G'	8	heated	0.004				
"	8	unheated	0.239	0.235	1.27	0.11	0.014
peak H'	8	heated	0.002				
"	8	unheated	0.242	0.240	1.30	0.08	0.010

Table IV. The protein content of peak A and various fractions obtained from it by DEAE-cellulose column chromatography at pH 8.2.

Fraction	ml. used in assay	Absorbance at 650 mu	mg of protein per ml
none	0	0	0
peak A	1.0	0.408	0.112
peak A'	1.0	0.083	0.018
peak B'	1.0	0.058	0.010
peak C'	1.0	0.086	0.019
peak D'	1.0	0.076	0.016
peak E'	1.0	0.079	0.017
peak F'	1.0	0.060	0.011
peak G'	1.0	0.096	0.021
peak H'	1.0	0.070	0.014

fractions which exhibit significant inhibition are found in peak A and peak A'. The fold of purification of peak A' fraction can be calculated as follows:

$$\text{Fold of purification} = \frac{0.058}{0.018} \div \frac{0.112}{0.108} = 3.4$$

Additional amounts of peak A' fraction were collected by chromatographic separations of additional portions of the peak A fraction. The peak A' fractions were combined and the solution was dialyzed, concentrated in dialysis tubing hanging in front of an electric fan and lyophilized. The lyophilized preparation was used to study the nature of the inhibitor.

Nature of Inhibitor

Glycoprotein.

The possibility of carbohydrate being present in the isolated inhibitor was tested by the method of Dubois et al (43). One ml. of 5 percent phenol was added to one ml. of inhibitor solution containing 0.5 mg of isolated inhibitor. Five ml. of 96 percent sulfuric acid then were added with constant mixing. After ten minutes, the tube again was shaken and placed in a water bath at 25-30° C. for 20 minutes. Carbohydrate presence is indicated by an orange color which develops in the reaction mixture. The lyophilized peak A' fraction was found to contain carbohydrate by this test. It may be present as glycoprotein, since other inhibitors have been shown to be glycoprotein.

Thermostability.

A solution was prepared containing 1.5 mg./ml. of lyophilized inhibitor. The solution was heated at 98° C. in a water bath. A water-cooled condenser was attached to the flask to prevent evaporation. Six ml. samples were withdrawn after 0, 0.5, 1, 3, 5, and 7 hours of heating. Four ml. of each solution were used for inhibitory assay after the solutions were cooled to room

temperature. All solutions were assayed simultaneously by the method described earlier. Data are shown in Table V. From this table, it is obvious that the isolated inhibitor is slowly heat labile.

Type of Inhibition.

The inhibition was studied by the double reciprocal method of Lineweaver et al (44) in an effort to determine the type of inhibition. In this method the inhibitor concentration is held constant and the inhibitory effect is measured by varying the substrate concentration. Thus, 100 ml. of 5 percent casein in 0.107 M phosphate buffer pH 8.4 was prepared and adjusted to pH 8.4 with dilute sodium hydroxide solution. Solutions containing 4, 3, 2, 1, and 0.5 percent casein then were prepared by appropriate dilution. The remainder of the experiment was carried out as described earlier, except that the reaction time used in this study was 2.5 hours instead of 4 hours.

The experimental data are shown in Table VI. From these values 1/S and 1/V were calculated, and a plot was prepared as shown in Fig. 5 according to the double reciprocal method of Lineweaver et al. It was concluded that the inhibition was non-competitive.

Homogeneity.

The homogeneity of the lyophilized peak A' fraction was investigated by gel electrophoresis as described above. There were two bands on the electrophoresis gel, as shown in Plate II. This indicates that the isolated inhibitor still was not homogeneous.

DISCUSSION

Although the purified inhibitor preparation contains carbohydrate, it possibly may be present as an impurity rather than as part of the inhibitor.

Table V. Effect of heating on activity of inhibitor

Heating time in hours	ml. of inhibitor used in assay	Trypsin solution	Absorbance at 620 mμ	Percent inhibition
0	0	heated	0	
"	"	unheated	0.248	
"	4	heated	0	
"	"	unheated	0.152	39
0.5	"	unheated	0.202	19
1	"	unheated	0.221	11
3	"	unheated	0.242	2
5	"	unheated	0.237	4
7	"	unheated	0.243	2

Table VI. Effects of inhibitor and substrate concentrations on hydrolysis of casein by trypsin.

	Inhibitor absent						Inhibitor present					
Percent casein concentration (S)	5	4	3	2	1	0.5	5	4	3	2	1	0.5
Absorbance at 620 mu after 2.5 hours of hyfrolysis (V)	.308	.278	.226	.175	.106	.061	.185	.180	.162	.135	.082	.048
1/S	.20	.25	.33	.50	1	2	.20	.25	.33	.50	1	2
1/V	3.2	3.6	4.4	5.7	9.4	16.4	5.4	5.6	6.2	7.4	12.2	20.8

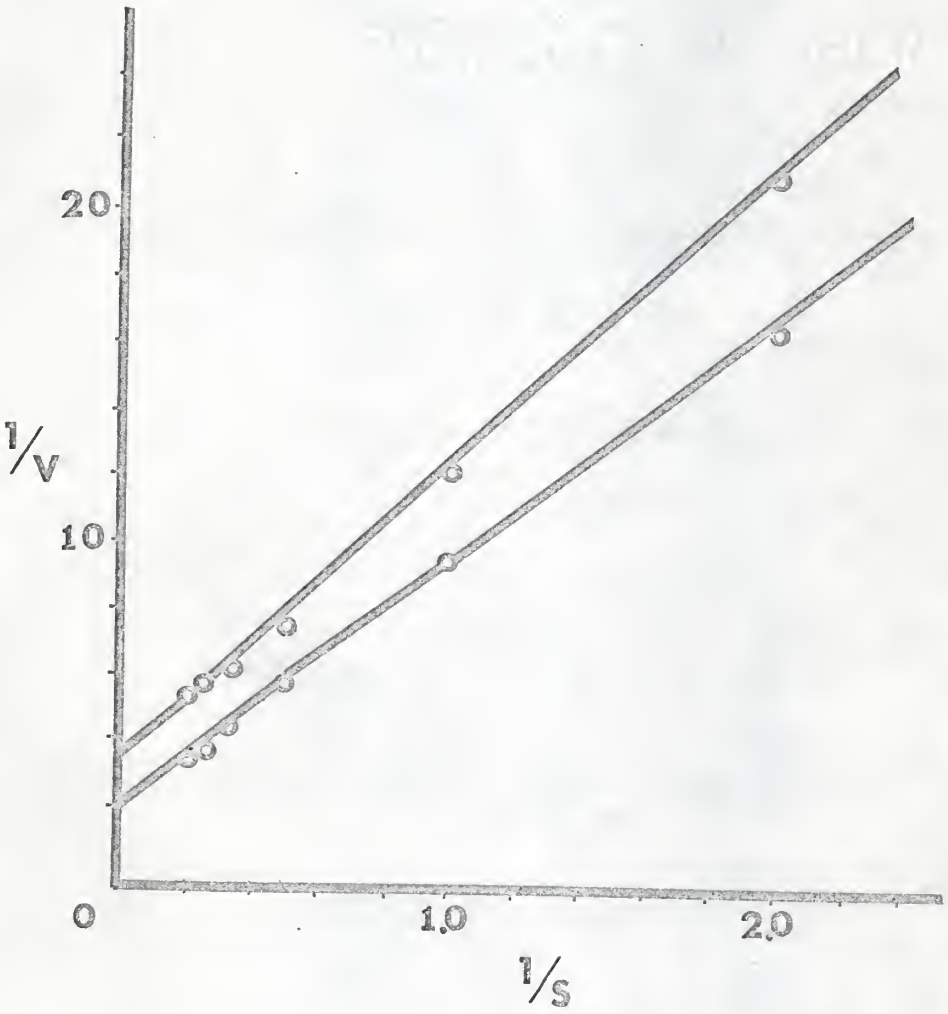

Fig. 5. Double reciprocal plot of the data of Table VI.

EXPLANATION OF PLATE II

Disc electrophoresis pattern of peak A'.

PLATE II

this might be studied by sephadex gel filtration whereby components of differing molecular weights can be separated. It would be necessary to measure absorbance of the fractions at 280 mu and to test them for the presence of carbohydrate. By comparing the patterns of these two measurements during elution, it should be possible to gain information regarding the glycoprotein nature of the inhibitor.

It was shown by gel electrophoresis that the isolated inhibitor was not homogeneous. It is not known which of the bands on the gel column is the inhibitor. This problem might be solved by performing electrophoresis with several large columns under comparable conditions. One column could be stained to determine the positions of the components. Using the stained column as a guide, the others could be sliced to separate the two components. Each component then could be elute and assayed for inhibitory activity. Multiple columns probly would be neeessary to obtain enough of each fraction for an inhibitory assay.

Nothing is known about a relationship between plant growth and inhibitor concentration. Hence, further studies of the inhibitor also should include its distribution in the plant and changes in inhibitory activity as the plant develops. Ultimately, a sufficient amount of the inhibitor should be isolated that its <u>in vitro</u> activity can be determined by controlled animal growth experiment.

SUMMARY

A study was made of a trypsin inhibitor in the vegetative portion of alfalfa. Alfalfa meal was extracted with acetone and ethanol to remove lipids and chlorophyll. The inhibitor then was isolated by extraction with acidic

water and precipitation with ammonium sulfate. It was purified further by DEAE-cellulose chromatography.

A double reciprocal study indicated the substance to be a non-competitive inhibitor. It was inactivated slowly by heating in aqueous solution. Its electrophoretic behavior and its positive reaction both with Folin reagent and phenol-sulfuric acid indicated it to be a polypeptide-carbohydrate complex or a glycoprotein.

ACKNOWLEDGMENTS

The author wishes to thank his major professor, Dr. H. L. Mitchell, for his constant assistance and constructive criticism throughout this work, and to express his sincere gratitude and thanks to Dr. R. E. Clegg for his advice and help.

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ISOLATION AND CHARACTERIZATION OF A TRYPSIN INHIBITOR OF ALFALFA

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AN ABSTRACT OF A MASTER'S THESIS

Submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1969

It has been demonstrated that aqueous extracts of fresh alfalfa forage inhibit the digestion of casein by trypsin *in vitro*, and that a similar inhibition of trypsin is observed by extracts of commercially dehydrated alfalfa. The inhibitor seems to withstand the heat during the process of dehydration. An effort was made in this investigation to devise a procedure for isolation of the inhibitory substance and to study its chemical nature and mode of action.

The inhibitory activities of extracts of alfalfa were determined by measuring the decrease in the amount of amino acids released during the *in vitro* hydrolysis of casein by trypsin when the extracts were added. Amino acids were determined by complex formation with copper by adding copper phosphate suspension to the deproteinized hydrolysate. The blue amino acid-copper complex was measured at 620 mu spectrophotometrically.

The protein content of inhibitor was determined by the Folin-Lowry method. The color formed by the Folin-Lowry reagent is due to the reaction of protein with alkaline copper in the reagent and to the reduction of phosphomolybdate and phosphotungstate in the reagent by the tyrosine and tryptophan of the protein. The intense blue color formed was measured at 650 mu with a Beckman DU Spectrophotometer.

The procedures involved in the isolation of inhibitor were as follows: Alfalfa meal was extracted with acetone and ethanol to remove lipids and chlorophyll. The inhibitor then was isolated by extraction with acidic water and precipitation with ammonium sulfate. It was further purified by DEAE-cellulose column chromatography.

The homogeneity of the isolated inhibitor was investigated by means of disc electrophoresis. The possibility of carbohydrate being present in the

isolated inhibitor was studied with the phenol-sulfuric acid carbohydrate test. Experimental results showed that the isolated inhibitor was not homogeneous and that it contained carbohydrate.

The type of inhibitor was studied by the double reciprocal method of Lineweaver et al. It was concluded that the inhibition was non-competitive. A thermostability study showed that the inhibitor lost its inhibitory activity gradually when it was heated in aqueous solution. Its electrophoretic behavior and its positive reaction both with Folin reagent and phenol-sulfuric acid indicated it to be a polypeptide-carbohydrate complex or a glycoprotein.